Chlorophyll and Carotenoid Content of *Dunaliella salina* at Various Salinity Stress and Harvesting Time

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Abstracts. The Green halophylic species microalgae *Dunaliella salina* normally lives in hypersaline waters. The pigments of this algae (chlorophyll and carotenoids) have potential as nutraceutical products because of their bioactivity as natural antioxidants. This study was aimed to determine the effect of salinity stress and harvesting time on the production of chlorophyll (*a* and *b*), and carotenoids. *Dunaliella salina* was cultured on a laboratory scale at various levels of salinity, namely 20, 25, 30, 35, and 40 ‰, respectively. Pigment were harvested 3 times, namely during peak of logarithmic growth (P0), 24 hrs after peak (P1), and 48 hrs after peak (P2). The culture media was enriched with Walne's solution, at pH 7 under 35 µmol quata. $m^{-2}.s^{-1}$ light intensity continuously at room temperature. The results showed that *D. salina* grew at given salinity. The highest and lowest growth was achieved at salinity 30 ‰ and 40 ‰, respectively. The highest chlorophyll *a* and *b* production was achieved at salinity 20 ‰ and at harvesting time the day after peak growth, while the highest production of carotenoid was obtained at salinity 25 ‰ harvested 48 hrs after the peak of growth. In conclusion, salinity stress was effective to increase chlorophyll and carotenoid production of *D. salina.*

1. Introduction

Marine microalgae are a source of natural compounds that are rich in benefits. The compound is in the form of metabolites that can be used as raw materials for the pharmaceutical, food and cosmetics industries, one of which is biopigment (organic pigment). Biopigment can be applied as a natural food coloring, including chlorophyll (as a green coloring) and carotenoids (as red, orange yellow coloring) [1]. Carotenoid chlorophyll is an organic pigment (biopigment) found in chloroplast and other organelles in plants including *Dunaliella salina* (Chlorophyta) which are potential to produce these chlorophylls, especially carotenoids.

Chlorophyll is a commercially natural green pigment, it has been applied in the food, cosmetics and pharmaceutical industries. Chlorophyll is consumed as nutraceutical because of its antioxidant, anti-inflammatory, antimutagenic, and antibacterial functions [2]. Biopigment (especially carotenoids) is a potential commercial product. Microalgae is the most important and non-toxic source of natural carotenoids. Carotenoids in microalgae cells serve as a protective oxidative stress associated with respiratory metabolism that produces free radicals [3][4]. The demand for natural carotenoids in the global market is enormous for various uses in the fields of food nutrition, food coloring, medicines, and cosmetics along with consumer awareness of natural ingredients and health [5].

Microalgal biopigment is often detected in very low concentrations during optimum conditions of culture. This become economic constraints in mass production commercially. Some Chlorophyta require special treatment to increase primary and secondary biopigment production, such as giving excessive light intensity and high salinity treatment [6]. *Dunaliella salina* is a green algae that is able to live in a large range of salinity, starting under 0.5 M (≈30 ‰) until saturated salinity level [1]. Several studies on *D. salina* cultivation have been carried out. According to Khatoon et.al.[6], salinity affected growh and proximate significantly. The highest growth was achieved in the media salinity of 10 ‰, when compared to 30 and 50 ‰, as well as the levels of protein, lipids and carbohydrates. *D. salina* was producer for caretenoid family pigments such as β-carotene, lutein, and zeaxanthin [7]. Studied by Mofeed [8], revealed that light intensity and salinity affected the production of chlorophyll and carotenoidsn[. The highest cell density and carotenoid content were achieved in high intensity light conditions with 2 M salinity (≈117 ‰), while the highest chlorophyll production was obtained at 1M salinity (≈58.5 ‰). Similar study also revealed that the highest cell density, chlorophyll content, and carotenoids were obtained at salinity of 30 ‰ [9]. Yet another study suggested that the highest cellular density was obtained at salinity of 30 ‰, but the highest carotenoid content was obtained at 35 ‰ [10]. Many factors have been known to influence microalgae pigment production, i.e: nutrients, salinity, pH, temperature, and light intensity. Abiotic stress can be used to trigger increased metabolite production. Pigment production (especially carotenoids) was affected by salinity, light, and nutrients [11]. Salinity was a major limiting factor for plant propagation because it can inhibit central metabolic activities such as photosynthesis [12]. This was the basis of this study, which determines the effect of different media salinity on *D. salina* biopigment production.

2. Research Methods

2.1 Dunaliella salina and cultivation

Microalgae *Dunaliella salina* was obtained from the Brackish Water Aquaculture Development Center (BBPAP), Jepara. Media culture was steriled sea water enriched with Walne's solution with several modifications [9]. Fifteen glass containers were set up to batch culture microalgae with 3 L of sterilled seawater enriched with Walne solution in each glass. Each container was inoculated with microalgae at initial density of 0.60 x 10^6 cells.mL^{-1}. Salinity was kept as treatment, namely: 20, 25, 30, 35, and 40 ‰, respectively. Moderate aeration was applied continuously, while illumination at 35 µmol quanta.m^{-2}.s^{-1} with 20 watts of TL was given for 24 hours. Culture conditions were maintained at room temperature (24 ± 1ºC) with pH 7. All cultures were made in 3 replicates. Preliminary cultivation was carried out to investigate *D. salina* growth patterns at different salinity

2.2 Determination of D. salina biomass

Microalgae were cultured for 6 days. Harvesting of *D. salina* biomass were carried out at the end of exponential growth, namely PO, 24 hours after P0 (namely P1), and 48 hours after P0 (namely P2). Cellular growth were measured by means of haemocytometer. Total biomass achieved were compared in correspond to different salinities and harvesting time. Five mL of algae biomass were taken, then extracted at 1008 g for 15 minutes. Wet gained pellet (±1g) were then macerated with 1 mL of 100 % pure aceton. Homogenazing was carried out by means of vortex mixer for 1-2 minutes, prior to incubation for 24 hours at refrigerator and recentrifugation to get supernatant. Absorbant of supernatant was measured spectrophotometrically. Biopigment extracted in acetone solvent was calculated using the Lichtenthaler and Buschmann equation[13] and converted to units of µg.g^{-1} of wet biomass as follows:

$$c_a\,(\mu g/ml) = 11.24\,A_{661.6} - 2.04\,A_{644.8}$$

$$c_b\,(\mu g/ml) = 20.13\,A_{644.8} - 4.19\,A_{661.6}$$

$$c_{(x+c)}\,(\mu g/ml) = \left(1000\,A_{470} - 1.90\,c_a - 63.14\,c_b\right)/214$$

Notes: C_a = chlorophyll *a;* C_b = chlorophyll *b;* $C_{(x+c)}$ = total carotenoid

2.3 Statistical analysis
All data were analyzed statistically with Manova Repeated Measures. Any signicantly data obtained ($P \leq 0.05$) were evaluated from *P values*.

3. Results and Discussions

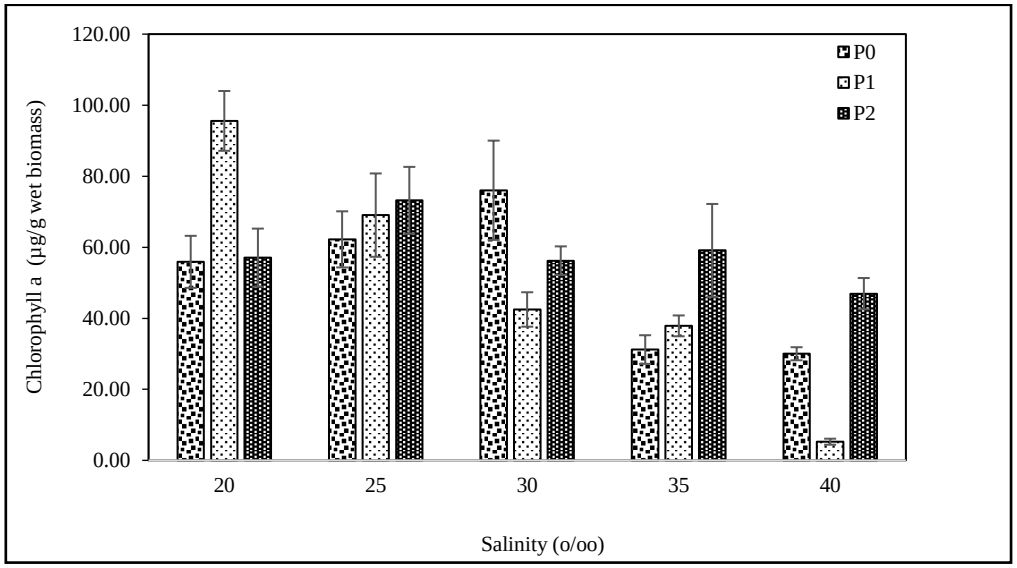
Six days preliminary experiment at initial density of 0.60 x 10^6 cell. mL^{-1} reached peak of logarithmic phase at day 4 for all cultures. The highest cellular density was achieved at salinity 30 ‰, at P0, meanwhile the lowest was abtained at salinity of 40 ‰ 48 hrs after reaching peak (P2) (Table 1).

Table 1. Cellular density (μ ±SD, n=3; x10^6 cells.mL^{-1}) of *D. salina* cultured under different salinities and harvesting times

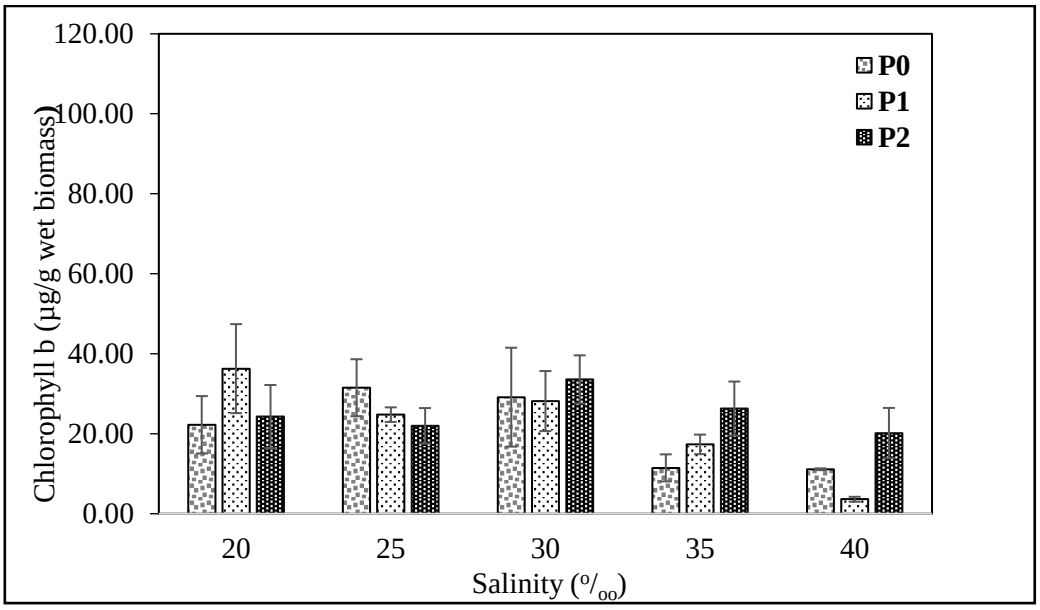
Salinity (‰)	Harvesting Time		
	P0	P1	P2
20	1.231±0.025	1.123±0.030	0.864±0.031
25	0.994±0.010	0.976±0.018	0.965±0.008
30	1.259±0.014	1.222±0.027	1.186±0.000
35	0.832±0.025	0.818±0.024	0.808±0.0241
40	0.892±0.005	0.869±0.013	0.858±0.011

Notes:
P0 = Harvesting Time at the end of exponential phase
P1 = Harvesting Time 24 hrs after P0
P2 = Harvesting Time 48 hrs after P0

As can be seen from Figure 1 (a, b), the highest content of chlorophyll *a* and *b* was obtained at 24 hrs after peak (P1) at salinity 20 ‰. Chlorophyll *a* content was 95.57±8.42 μg.g^{-1} (Fig. 1a) and chlorophyll *b* was 36.28±11.13 μg. g^{-1}wet weight (Fig. 1b). Meanwhile, the lowest content of chlorophyll *a* (5.26±0.85 μg. g^{-1}wet weight) and chlorophyll *b* (3.62±0.61 μg. g^{-1}wet weight) was obtained at 24 hrs after peak (P1) at salinity 40 ‰.



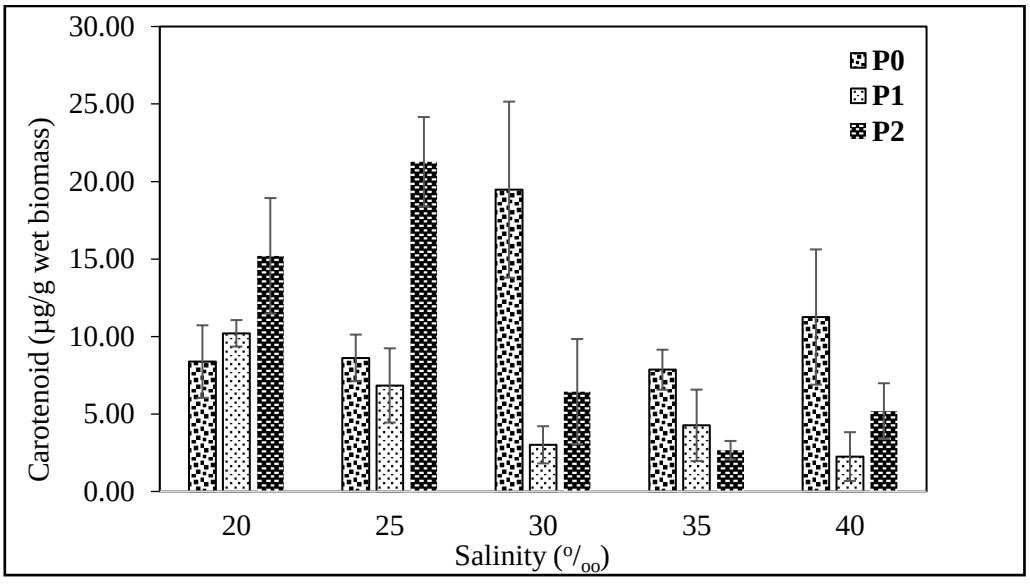
(a)



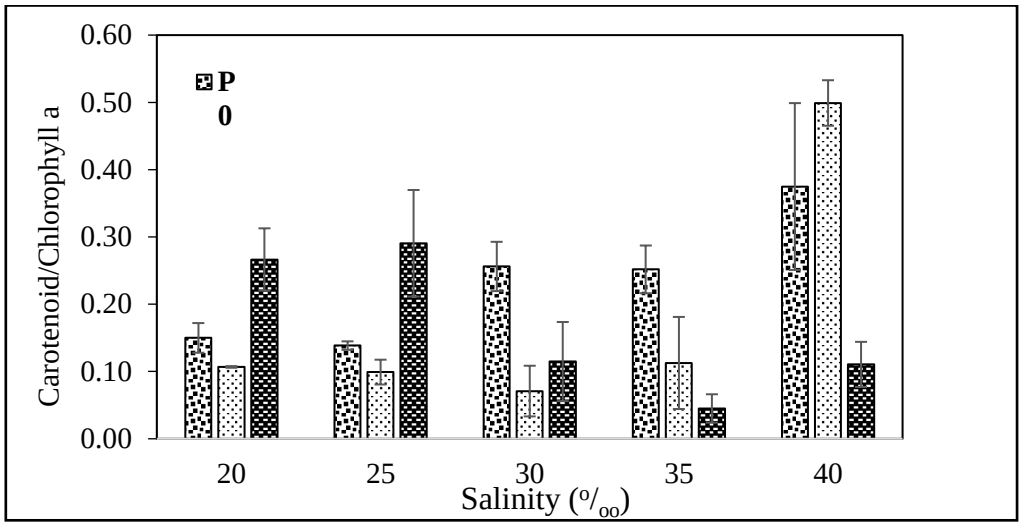
(b)

Figure 1. Content of chlorophyll *a* (a) dan *b* (b) in *D. salina* cultured under different salinities and harvesting time. P0 (harvesting time at peak of log phase, P1 (harvesting time 24 hrs after P0), P2 (harvesting time 48 hrs after P0)

Carotenoid production was not in line with chlorophyll production and peak growth. The highest carotenoid level (21.28 ± 2.88 µg. g^{-1} wet biomass) was achieved in salinity 25 °/oo at 48 hrs after peak (P2), while the lowest (2.25 ± 1.58 µg. g^{-1} wet biomass) was gained at salinity 40 °/oo ,24 hrs after peak (P1). The highest chlorophyll *a* to carotenoid content ratio (0.50 ± 0.01µg. g^{-1} wet biomass) was achieved at salinity 40 °/oo, 24 hrs after reaching peak (P1).



(a)



(b)

Figure 2. Content of caretenoids (a) dan caretenoid to chlorophyll a ratio (b) in D. *salina* under different salinities and harvesting time. P0 (harvesting time at peak of log phase, P1 (harvesting time 24 hrs after P0), P2 (harvesting time 48 hrs after P0).

Statistical analysis by Manova Repeated Measure revealed that salinity significantly influenced to cellular density, chlorophyll *a* and *b* content, carotenoids, as well as the ratio of carotenoids to chlorophyll *a* . Time to harvest also influenced to all examined variables except for chlorophyll *b*. The interaction of the two variables also very significantly affected on biomass and pigment production (Table 2)

Table 2. Manova repeated measure analysis of the responses of all examined variables

Response	Salinity			Harvesting Time			Salinity*Harvesting Time		
	Df	F values	P values	Df	F values	P values	Df	F values	P values
Density Cell	4	263.44	0.000***	2	169.97	0.000***	8	18.73	0.000***
Chlorophyl a	4	41.59	0.000***	2	5.61	0.012*	8	18.03	0.000***
Chlorophyll b	4	7.61	0.004**	2	2.29	0.127	8	4.55	0.003**
Carotenoid	4	6.79	0.007**	2	30.06	0.000***	8	20.70	0.000***
Carotenoid/ Chlorophyll a	4	423.18	0.000***	2	275.24	0.000***	8	430.08	0.000***

(*) indicates P≤0.05, (**) P≤0.01,(***)P P≤0.001

The study obtained the best biomass production of microalgae at salinity30 °/oo when harvested at peak of log phase (P0). Meanwhile, content of chlorophyll a and b was highest at 20 °/oo and harvesting time at peak (P0). The highest carotenoids content was obtained at culture condition of salinity 25 °/oo,at harvesting time 48 hrs after peak (P2). The highest content of both chlorophyll a and b at salinity 20 °/oo was pressumed due to culture condition was suitable fo microalgae to run optimum photosynthesis. The role of chlorophyll is to capture radiant energy in form of photon in light reactions

(photolysis) in thylacoid. Results of this light reaction in the form of energy-rich compounds (ATP and NADPH) will later be used to drive the dark reaction (Calvin cycle) in the stroma, which assimilates CO_2 into glucose. When salinity was increased to 25 ‰, chlorophyll production decreased, thus triggering *D. salina* to produce carotenoids (carotenogenesis) to help chlorophyll capture photons while protecting chlorophyll. Photosynthetic reactions can still run up to 30 ‰, where the highest cell density was reached. Chlorophyll and carotenoids decreased in production at salinity of 35 and 30 ‰, so photosynthesis was even decreasing. As a result, more cells were dying and in turn decreasing cell density.

Carotenoids in *D.salina* are relatively similar to high plants [5]. This very widely strains of microalgae has ability to accumulate carotenoids and optimize their capacity as photoprotection also varies [7]. The results of other studies on the effect of salinity on carotenoid production (fucoxanthin) on some halotolerant marine microalgae (optimum salinity> 50 ‰) and not halotolerant (optimum salinity <50 ‰) proved that there was a positive inversely proportional to chlorophyll a levels, and the ratio of carotenoids to chlorophyll a increases with increasing salinity. When the salinity was 40 ‰, the ratio was the highest that was up to 50%. This proves that the role of carotenoids as accessory pigments is relatively large, so the photosynthetic reaction can continue. Increased salinity increased the ratio of carotenoids to *D. salina* chlorophyll microalgae [14].

The ability of *D. salina* to increase photosynthetic activity in high salinity is remarkable, because in most plants and cyanobacteria, it actually inhibits photosynthesis. Increased enzyme production to produce ATP and redox energy indicates an increase in metabolic activity when salinity increases, light reactions increase to 150% and darkness is 120% [12]. The highest chlorophyll production was at 20 ‰, but the highest cell density was at 30 ‰. Thus, increased salinity may triggers increased photosynthetic activity and was thought to also increase the biosynthesis of metabolites needed for cell growth. When nitrogen (N) nutrients are still available, biosynthesis of nitrogen compounds such as chlorophyll and protein is enhanced. Protein is one of the constituents of cells that are important for growth. Conversely, when N decreases biosynthesis of starches and lipids is increased, including carotenoids (lipophilic pigments). The purpose of this biosynthesis is as a source of energy for the survival of microalgae through respiration, due to decreased biosynthetic activity. Study of carotenogenesis [15] proved that the increase in salinity and limited nitrogen source media affected the process.

Production of chlorophyll *a* and *b*, biomass, as well as concentration K^+, Ca^{2+}, and Mg^2 in cytoplasm decreases as salinity increase [16]. Salinity stress triggers the production of proline and nitric oxide (NO) which is a signal to produce chemical compounds that are responsible for salinity tolerance [11, 17]. Microalgae respond to abiotic stress, including salinity by regulating the production of its metabolites. Compounds that are closely related to the mechanism of salinity tolerance are glycerol. Every strain has a different behavioral and physiological response to salinity pressure. Increased photosynthesis in response to increased salinity produces energy-rich ATP and NADH. Energy not only produces primary metabolites (especially starch), but also produces secondary metabolites as an effort to adapt to the environment, such as glycerol [18]. Glycerol as an osmotic element, is responsible for the survival of the condition to be hyposaline or hypersaline. Starch degradation plays role as an energy source as well as for glycerol biosynthesis. Starch levels are negatively correlated with glycerol. When conditions become hyposaline, glycerol becomes a lipid. *D. salina* cells are able to reproduce in hypersaline media, but the NaCl concentration in intracellular fluid is maintained at low concentrations [19]. Biochemical arrangements control glycerol production in chloroplasts and their degradation. There are 3 enzymes involved, NADPH-dihydroxyacetone-reductase, dihydroxyacetone kinase and glycerol-1-phosphatase. Glycerol is synthesized as an attempt at intracellular osmotic pressure when salinity stress occurs. Production increases when salinity is 2 M (≈117 ‰), while maximum growth has occurred at salinity of 0.5 M (≈117 ‰)[20]. When salinity is high, starch degradation occurs and a decrease in carbon flow enters the Kreb's cycle (tricarboxylic acid cycle) .The source of carbon is used for glycerol biosynthesis [21].

More microalgae protein and chlorophyll were produced during the log phase, while carotenoids and lipids were higher in the stationary phase. In addition, *D. salina* accumulated more carotenoids at the end of the stationary phase [22,23]. Variation in harvesting time also affected cell growth or density and pigment production. The highest cell density was reached at the peak of the log phase (P0), while the highest levels of chlorophyll *a* and *b*, was attained at the following day (P1) and the highest level of carotenoid was gained 2 days after the peak of the log phase (P2). During log phase, microalgae may produce more primary metabolites for their survival, so biomass is now at its peak. Unlike the case with secondary metabolites, these compounds are only recently produced when entering the stationary phase. Chlorophyll and carotenoid pigments can be categorized as secondary metabolites when they function as cell antioxidants, and have been shown to be high when harvested during the stationary phase.

4. Conclusions

Salinity and harvesting time affected growth, content of chlorophyll and carotenoids of *Dunaliela salina.* Carotenoid production increased when chlorophyll production decreased. The highest biomass of algae was achieved at salinity 30 ‰ (peak log phase). Meanwhile, the highest content of chlorophyll was attained at salinity 20 ‰ at stationary phase. Similar to chlorophyll, the highest carotenoid was gained also at stationary phase at salinity 25 ‰. The method of microalgal cultivation with salinity manipulation at different harvesting time can be a reference for increasing biopigment production for commercial purposes.

References.

[1] Mulders K J M, Lamers P P, Martens D E and Wijffels R H 2014 Phototrophic Pigment Production with Microalgae: Biological Constraints and Opportunities. *Journal of Phycology* **50(2)** 229–42.

[2] Ferreira VS and Anna C S 2017. Impact of Culture Conditions on the Chlorophyll Content of Microalgae for Biotechnological Applications. *World Journal of Microbiology and Biotechnology,* **33(1)** 1–8.

[3] Raposo M F J, De Morais A M M B and R M S C De Morais 2015. Carotenoids from Marine Microalgae: A Valuable Natural Source for the Prevention of Chronic Diseases. *Marine Drugs*, **13(8)** 5128–55.

[4] Hu J, Nagarajan D., Zhang Q, Chang J S and Lee D J 2018. Heterotrophic Cultivation of Microalgae for Pigment Production: A Review. *Biotechnology Advances,* **36(1)** 54–67.

[5] Ye Z W, Jiang J G and Wu G H 2008. Biosynthesis and Regulation of Carotenoids in Dunaliella: Progresses and Prospects. *Biotechnology Advances,* **26(4)** 352–60.

[6] Khatoon H, Haris N, Banerjee S, Rahman N A, Begum H, Mian S., Abol-Munafi A B, and Endut A 2017. Effects of Different Salinities on the Growth and Proximate Composition of *Dunaliella* sp. Isolated from South China Sea at Different Growth Phases. *Process Safety and Environmental Protection,* **112** 280–87.

[7] Xu Y, Ibrahim I, Wosu C, Ben-Amotz A and Harvey P 2018. Potential of New Isolates of *Dunaliella salina* for Natural β-Carotene Production. *Biology,* **7(1)** 14.

[8] Mofeed J 2015. Effects of Salinity and Light Intensity on Production of ß -Carotene and Glycerol from The Halotolerant Alga *Dunaliella salina* (Chlorophyta) Isolated from Zaranik Nature Reserve, North Sinai, (Egypt), *Egypt. J. Exp. Biol. (Bot.)*, **11(1)** 21–29.

[9] Zainuddin M, Hamid N, Mudiarti L, Kursistyanto N, and Aryono B 2017. Pengaruh Media Hiposalin dan Hipersalin Terhadap Respon Pertumbuhan dan Biopigmen *Dunaliella salina*. J*urnal Enggano,* **2(1)** 46–57.

[10] Mahardani D 2017. Pengaruh Salinitas Berbeda Terhadap Pertumbuhan dan Kandungan Karotenoid *Dunaliella* sp. dalam Media Ekstrak Daun Lamtoro (*Leucaena leucocephala)*. *Jurnal Perikanan dan Kelautan*, **7 (1)** 50–58.

[11] Paliwal C, Mitra M, Bhayani K, Bharadwaj SVV, Ghosh T, Dubey S and Mishra S 2017. Abiotic Stresses as Tools for Metabolites in Microalgae. *Bioresource Technology,* **244** 1216–26.

[12] Liska A J 2004. Enhanced Photosynthesis and Redox Energy Production Contribute to Salinity Tolerance in Dunaliella as Revealed by Homology-Based Proteomics. *Plant Physiology*, **136(1)** 2806–17.

[13] Lichtenthaler H.K and Buschmann C 2001. Chlorophylls and Carotenoids : Measurement and Characterization by UV-VIS Spectroscopy. *Current Protocols in Food Analytical Chemistry,* F4.3.1-F4.3.8

[14] Aguilar-Machado D E, Benavente-Valdés J R, Méndez-Zavala A, and Montañez J C 2015. Effect of Salt-Stress on the Production of Pigments by *Chlorella sorokiniana* under Photoheterotrophic Culture. XVI Congreso Nacional de Biotecnologia y Bioingenieria, 21-26 Juni 2015. Guadalajara, Mexico.

[15] Shang C, Wang W, Zhu S, Wang Z, Qin L, Alam MA., Xie J and Yuan Z 2018. The Responses of Two Genes Encoding Phytoene Synthase (Psy) and Phytoene Desaturase (Pds) to Nitrogen Limitation and Salinity up-Shock with Special Emphasis on Carotenogenesis in *Dunaliella parva*. *Algal Research,* **32** 1–10.

[16] Gomes M A C., Pestana I A, Santa-Catarina C, Hauser-Davis R A., and Suzuki M S 2017. Salinity Effects on Photosynthetic Pigments, Proline, Biomass and Nitric Oxide in *Salvinia auriculata* Aubl. *Acta Limnologica Brasiliensia,* **29** 1–13.

[17] Fang L, Qi, S, Xu Z, Wang W, He J, Chen X and Liu J 2017. De Novo Transcriptomic Profiling of *Dunaliella salina* Reveals Concordant Flows of Glycerol Metabolic Pathways upon Reciprocal Salinity Changes. *Algal Research,* **23** 135–49.

[18] Charioui I, Chikhaoui M, El Filali F M, Abbassi F.M, Banaoui A and Kaaya A 2017. Production in Cell Biomass and Carotenoids under the Effect of a Saline Stress in Microalgae *Dunaliella* spp. Isolated from Moroccan Saharian Saline. *Int.J.Curr.Microbiol. App.Sci.*, **6(8)** 286–294.
[19] Chen X J, Wu M J,Jiang Y and Yan Y B 2015. *Dunaliella salina* Hsp90 Is Halotolerant. *International Journal of Biological Macromolecules,* **75** 418–425.
[20] Ben-Amotz, Sussman A I and Avron M 1982. Glycerol production by Dunaliella. *Cellular and Molecular Life Sciences CMLS.*, **38(1)** 49-52.
[21] Xia B B, Wang S H, Duan J B and Bai LH 2014. The Relationship of Glycerol and Glycolysis Metabolism Patway under Hyperosmotic Stress in *Dunaliella salina*. *Central European Journal of Biology*, **9 (9**) 901–8.
[22] Paes C R P S, Faria G R, Tinoco N A B, Castro D J F A, Barbarino E and Lourenço S O 2016. Growth, Nutrient Uptake and Chemical Composition of *Chlorella* sp. and *Nannochloropsis oculata* under Nitrogen Starvation. *Lat. Am. J. Aquat. Res.*, **44(2**) 275–92.
[23] Pasqualetti M, Bernini R, Carletti L, Crisante F and Tempesta S 2010. Salinity and Nitrate Concentration on the Growth and Carotenoids Accumulation in a Strain of *Dunaliella salina* (Chlorophyta) Cultivated under Laboratory Conditions. *Transitional WatersBulletin,* **4(2)** 94–104.